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Phosphorylation of BAD at Ser-128 during mitosis and paclitaxel-induced apoptosis

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Abstract Phosphorylation of BCL-2 family member BAD at different residues triggers different physiological effects, either inhibiting or promoting apoptosis. The recently identified phosphorylation site at Ser-128 enhances the apoptotic activity of BAD. We here show that BAD becomes phosphorylated at Ser-128 in the mitotic phase of the cell cycle in NIH3T3 cells. We also show that BAD-S128 is phosphorylated in taxol-treated mouse fibroblasts and MDA-MB-231 human breast cancer cells. However, expression of a phosphorylation-defective dominant negative BAD mutant did not block taxol-induced apoptosis. These data support the view that the phosphorylation of BAD Serine 128 exerts cell-specific effects on apoptosis. Whereas the BAD Serine 128 phosphorylation induces apoptosis in neuronal cells, it does not appear to promote apoptosis in proliferating non-neural cells during mitosis or upon exposure to the antineoplastic agent taxol.

Keywords: Apoptosis; BAD; Cell cycle; Taxol

1. Introduction

The proapoptotic BCL-2 family member BAD was the first cell death component to be identified as a regulatory target of survival signaling [1]. Active BAD induces apoptosis by inhibiting antiapoptotic BCL-2 family members, such as BCL-XL, thereby allowing two other proapoptotic members, BAK and BAX, to form complexes, leading to the release of cytochrome *c*, caspase activation, and apoptosis [2,3]. Survival signaling induces phosphorylation of BAD on Ser-112, Ser-136, and Ser-155, resulting in the binding of BAD to 14-3-3 proteins as an inactive complex [4].

It was recently reported that Cdc2 and JNK catalyze the phosphorylation of BAD at serine 128 in post-mitotic neuronal cells [5–8]. This led to BAD-mediated apoptosis by inhibiting BAD's sequestration by members of the 14-3-3 family of proteins. These findings provided a mechanistic explanation to apoptosis in neurons upon the suppression of neuronal activity or upon activation of the p75 neurotrophin receptor [5,7,9]. These findings also raised the major question of the role of the BAD Serine 128 phosphorylation in cells outside the nervous system. In particular, the finding that the mitotic kinase Cdc2 mediates the phosphorylation of BAD at Serine 128 in neurons [5] raises the question of whether the BAD Serine 128 phosphorylation occurs during mitosis in proliferating non-neural cells, a time in the cell cycle during which Cdc2 activity is high [10].

Agents such as vinca alkaloids and taxanes represent an important class of antineoplastic agents which are known to induce tumor cell apoptosis [11]. Although the mechanisms of apoptosis induction by these agents remain to be elucidated, Cdc2 and JNK have both been suggested to contribute to taxol-induced apoptosis in proliferating cells [12–14]. Here, we examined BAD-128 phosphorylation in dividing cells, and whether this type of phosphorylation is important for apoptosis induction by taxol.

2. Materials and methods

2.1. Materials

Paclitaxel and purvalanol were obtained from Sigma, SP600125 from Biomol Research Laboratories and cisplatin from Bristol-Myers Squibb.

2.2. Cells

MDA-MB-231 breast carcinoma, NIH-3T3 cells and MEF cell lines were maintained at +37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with fetal calf serum (10%), L-glutamate, penicillin and streptomycin.

2.3. Transfection procedure

Cells grown in 6 well plates were transfected for 4 h with a CK18 cDNA expression vector (kindly provided by Dr. Bisr Omary, Stanford University) and BADS128/BADS128A vectors using Lipofectamine2000 (Invitrogen). BAD vectors were fusions with GFP. After transfection, zVAD-fmk was added to inhibit apoptosis caused by the transfection procedure. After 24 h, cells were washed and paclitaxel was added and incubation was continued for another 24 h.

2.4. Assessment of apoptosis

Cytokeratin-18 cleavage was measured using reagents from PEVIVA AB (Bromma, Sweden). At the end of the incubation period with paclitaxel, NP40 was added to the tissue culture medium to a final concentration of 0.1% and an aliquot (500 μl) of the entire content of each well (from attached cells, floating cells and cell fragments, as well as activity released to the medium) was assayed for the CK18-Asp396 epitope by incubation with beads coated with an

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anti-CK18 antibody. The beads were washed and incubated with antibody M30 [15] which had been conjugated with HRP (obtained from PEVIVA AB).

2.5. Western blot analysis

Cell extract proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane for Western blotting.

2.6. Flow cytometric analysis

Cells were harvested, washed and fixed with 0.25% paraformaldehyde for 5 min. The cells were then washed and incubated with antibody in 100 μg/ml digitonin in PBS for 30 min. The antibody against BADSer128-P has been described [5], the antibody to GFP was obtained from Santa Cruz Biotechnologies (Santa Cruz, CA) and the antibody to BAD from Cell Signaling (Beverly, MA). After incubation, the cells were washed and incubated with FITC-conjugated secondary antibodies for 30 min, washed with PBS and analyzed by flow cytometry. CK18 cleavage was assessed using the monoclonal antibody M30 [15].

3. Results

3.1. Phosphorylation of Bad at Ser128 during mitosis

To characterize the phosphorylation of BAD Serine 128 in proliferating non-neural cells, we first determined if this phosphorylation event occurs in NIH-3T3 fibroblasts upon growth factor stimulation. NIH-3T3 cells were starved of serum and then stimulated with serum (10% FBS) for the indicated times in Fig. 1A. Serum stimulation of NIH-3T3 cells induced the phosphorylation of endogenous BAD at Serine 128, as determined by immunoblotting using an antibody that recognizes the Serine 128-phosphorylated form of BAD specifically [5] (Fig. 1A). The serum-induced phosphorylation of BAD occurred with delayed kinetics taking several hours to reach robust levels.

We next examined the phosphorylation of BAD in NIH-3T3 cells at the single cell level by immunocytochemical analysis. NIH-3T3 cells growing in 10% FBS were fixed and subjected to immunocytochemistry using the phospho-Serine 128 BAD antibody [5]. We found strong phosphoSerine 128 BAD immunoreactivity in cells undergoing mitosis but not in interphase cells (Fig. 1B). The specificity of the signal was demonstrated as the ability of the antibody to recognize endogenous phosphoSerine 128 BAD immunoreactivity was effectively competed with prior incubation with the phosphoSerine 128 BAD peptide but not with the unphosphorylated BAD Serine 128 peptide (Fig. 1B). Together, these results indicate that endogenous BAD undergoes phosphorylation at Serine 128 in proliferating fibroblasts. The tight association of the BAD Serine 128 phosphorylation with mitosis is consistent with the idea that BAD Serine 128 is a substrate of Cdc2 in proliferating cells.

We found little evidence of cell death in NIH-3T3 cells in which the BAD Serine 128 phosphorylation was detected as indicated by normal appearing nuclei that were stained with the DNA dye bisbenzimide (Hoechst 33258). These observations suggested that in contrast to neuronal cells in which the BAD Serine 128 phosphorylation triggers apoptosis [5,7–9], the phosphorylation of BAD Serine 128 in proliferating fibroblasts does not appear to correlate with apoptosis.

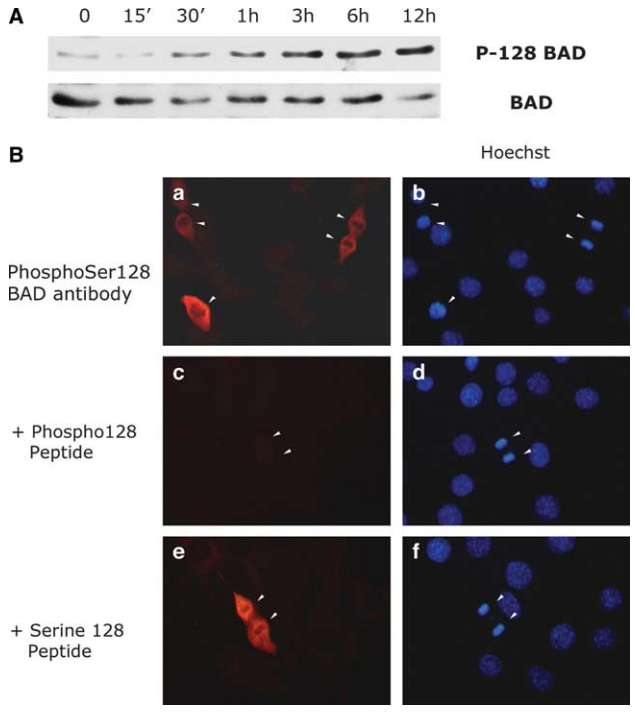

Fig. 1. Phosphorylation of endogenous BAD at Serine 128 in NIH-3T3 cells (A) 3T3 cells were starved of serum for 12 h, then 10% FBS was added to the media for the indicated time. Lysates of cells were subjected to immunoblotting with the phosphoSerine 128 BAD antibody (top panel) or an antibody that recognizes BAD regardless of its phosphorylation status (bottom panel). Serum stimulation induces the phosphorylation of BAD at Serine 128 with delayed kinetics. (B) NIH-3T3 cells growing in 10% FBS were fixed in 4% paraformaldehyde and subjected to immunocytochemical analysis with the phosphoSerine 128 BAD antibody and stained with the DNA dye bisbenzimide (Hoechst 33258). The phosphoSerine 128 BAD signal is shown in the left panels and Hoechst staining in the right panels. The phosphoSerine 128 antibody was incubated at 4 °C prior to immunocytochemistry with the phosphorylated Serine 128 BAD peptide (middle panels) or with the unphosphorylated Serine 128 BAD peptide (lower panels) as described [5]. Robust Serine 128 phosphorylated BAD immunoreactivity was detected in cells undergoing mitosis.

3.2. Phosphorylation of Bad at Ser128 following paclitaxel treatment

Having found that BAD undergoes phosphorylation at Serine 128 in growing fibroblasts during mitosis, we next determined if the BAD Serine 128 phosphorylation is induced in proliferating cells upon exposure to the antineoplastic agent taxol. Taxol is reported to induce apoptosis in part via the aberrant activation of the kinases Cdc2 and JNK [12–14], both of which have been implicated in the phosphorylation of BAD Serine 128 in neuronal cells [5,6]. Bad phosphorylation was examined in paclitaxel-treated cells after staining with the phospho-BAD-Ser128 antibody. Analysis by flow cytometry showed increased Ser-128 phosphorylation in paclitaxel-treated human breast cancer MDA-MB-231 cells (Fig. 2A and B) and mouse embryo fibroblasts (MEF) (Fig. 2C). Increased phosphorylation was also observed in taxol-treated MDA-MB-231 cells by Western blotting (Fig. 2D). The anticancer agent cisplatin, which induces apoptosis but not mitotic arrest, did not induce BAD Serine 128 phosphorylation. The JNK inhibitor SP600125 was found to partially inhibit BAD Ser 128 phosphorylation in MEF cells (Fig. 2C). The Cdc2

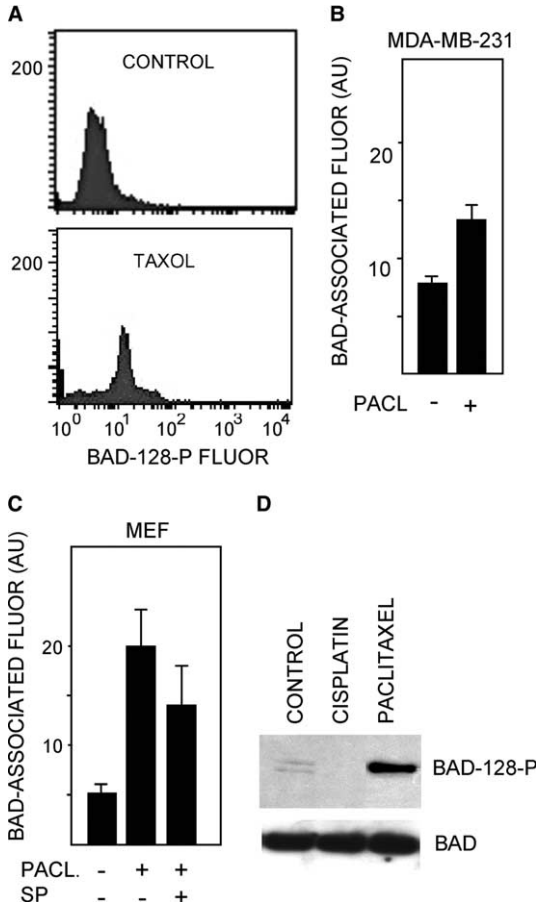

Fig. 2. Phosphorylation of Bad at Serine 128 in paclitaxel-treated cells. Human MDA-MB-231 cells (A, B) and mouse embryo fibroblasts (MEF) (C) were treated for 24 h with 500 nM paclitaxel, labeled with the phosphoSerine 128 antibody and analyzed by flow cytometry. SP = 10 μM SP600125. (D) Human MDA-MB-231 cells were treated with 500 nM paclitaxel or 20 μM cisplatin for 16 h and analyzed by Western-blotting using the phosphoSerine 128 antibody or an antibody to BAD. 80 μg protein was loaded on each lane.

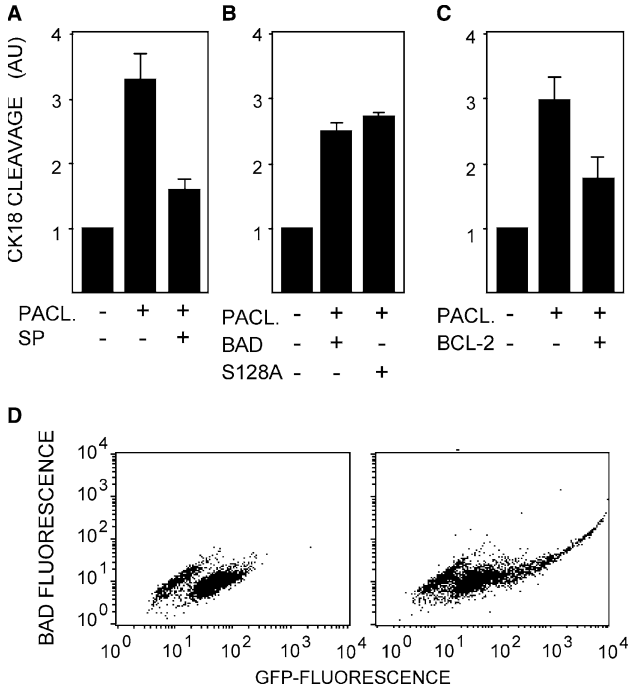

Fig. 3. Inhibition of paclitaxel-induced apoptosis by Bcl-2 but not by a dominant negative mutant of BAD. MEF cells were transfected with 2 μg of a CK18 cDNA expression vector and 2 μg of BAD cDNA expression vectors or 2 μg of a Bcl-2 cDNA expression vector. Paclitaxel was added 24 h after transfection and the levels of caspase-cleaved CK18 were assayed after an additional 24 h using the M30 antibody [16]. (A) Inhibition of CK18 caspase cleavage by SP600125 (10 μM). (B) CK18 cleavage in cells transfected with a BAD expression vector or an expression vector encoding a phosphorylation deficient dominant negative form of BAD (BADS128A). (C) Inhibition of CK18 caspase cleavage by a Bcl-2 expression vector. (D) Expression of BADS128A protein in transfected MEF cells. Cells were stained both for GFP (green fluorescent protein; expressed by the BADS128A construct) and BAD; the BADS128A vector is a GFP-fusion protein. Left: untransfected cells; right: cells transfected with BADS128A vector.

inhibitor purvalanol also inhibited BAD Ser-128 phosphorylation (data not shown). These data are consistent with the conclusion that both JNK and Cdc2 contribute to the phosphorylation of BAD at Serine 128 in taxol-treated proliferating cells. However, whether the negative effect of purvalanol on the phosphorylation of BAD at Serine 128 reflects a direct inhibitory effect of Cdc2 or due to secondary consequences of cell cycle inhibition is not clear.

3.3. Expression of a dominant BAD-S128A mutant does not block taxol-induced apoptosis

The finding that BAD is phosphorylated in mitotic and taxol treated cells raises the question of whether this phosphorylation is mechanistically important for taxol-induced apoptosis. We tested this possibility using a dominant negative mutant of BAD, BADS128A. Initial experiments using this mutant were not conclusive, however, due to difficulties to quantify apoptosis in the population of transfected cells. This was due to the presence of both post-mitotic micronucleated cells and apoptotic cells showing nuclear fragmentation. To solve these problems, we developed a cotransfection method based on an exogenous caspase-substrate (human CK18) that was introduced into MEF cells together with BAD expression vectors (see Section 2). CK18 caspase-cleavage was then assessed by a simple immunoassay [16]. Robust increases in the levels of caspase-cleaved CK18 were observed in paclitaxel-treated cells (Fig. 3A). Lower levels of caspase-cleaved CK18 were observed in cultures treated with paclitaxel in the presence of the JNK inhibitor SP600125 (Fig. 3A). MEF cells were cotransfected with CK18 cDNA and BADSer128A or with a Bad control cDNA expression vector. BADSer128A did not block caspase-cleavage of CK18 (Fig. 3B) over a range of different plasmid concentrations. As a control, we showed that transfection of Bcl-2 inhibits paclitaxel-induced CK18 cleavage (Fig. 3C). Expression of exogeneous GFP-BAD in transfected cells was verified by flow cytometry (Fig. 3D).

4. Discussion

In this study, we report the characterization of the BAD Serine 128 phosphorylation in proliferating non-neural cells. The phosphorylation of BAD at Serine 128, catalyzed by the

proline-directed kinases Cdc2 and JNK, promotes apoptosis in neuronal cells upon the suppression of neuronal activity or the activation of the p75 neurotrophin receptor [5,7,9]. Here, we report that the phosphorylation of BAD at Serine 128 also occurs in proliferating non-neural cells. The evidence provided in our study is consistent with the idea that both Cdc2 and JNK contribute to the phosphorylation of BAD at Serine 128 in proliferating cells. However, transfection of a BAD cDNA expression vector that cannot be phosphorylated at Serine 128 did not block taxol-induced apoptosis in proliferating cells. In addition, we were unable to demonstrate increased binding of Ser-128 phosphorylated BAD to Bcl-XL in taxol treated cells (M.H., unpublished data). Together, our results suggest that in contrast to the critical role of the BAD Serine 128 phosphorylation in inducing apoptosis in neuronal cells, the phosphorylation of BAD Serine 128 does not appear to promote cell death in proliferating fibroblasts. We interpret these results to suggest that phosphorylation of BAD at Serine 128 exerts distinct effects on apoptosis in neuronal cells and non-neural cells. In future studies, it will be important to determine the mechanisms that underlie the cell-specific nature of the biological effect of the BAD Serine 128 phosphorylation. The possibility remains that the phosphorylation of BAD at Serine 128 may serve roles in apoptosis in proliferating cells under specific circumstances. Given the importance of BAD in the death of thymocytes in response to DNA damage [17], it will be interesting to determine if the phosphorylation of BAD at Serine 128 plays any role in this situation. In addition, since BH3-only proteins may play roles in the cell outside of apoptosis, for example in regulation of the cell cycle [18], it will be interesting to determine if the BAD Serine 128 phosphorylation might impact on the cell cycle. In the future, it will be important to determine the function of BAD Serine 128 phosphorylation in different tissues using a mouse knock-in approach.

Mitogen activated kinase pathways are important for apoptosis induction by many stimuli, and are targets for combination therapy with taxol [19]. Many apoptotic signals cause activation of Jun-N-terminal kinase (JNK), and JNK activation has been linked to apoptosis [20]. Microtubuli inhibitors activate JNK in a variety of different cell lines [21,22], and inhibition of JNK protects cells from cytotoxicity induced by mitotic inhibitors [13]. Our results confirm previous findings of a pro-apoptotic role of JNK in paclitaxel-induced apoptosis [13].

The Cdc2 kinase has also been implicated in paclitaxel-induced apoptosis. Inhibition of this kinase using pharmacological inhibitors or anti-sense oligonucleotides results in apoptosis inhibition [12]. We found that the Cdc2 inhibitor purvalanol inhibits taxol-induced apoptosis (M.B., unpublished results). However, since purvalanol blocks the cell cycle, it might inhibit apoptosis by indirect mechanisms, rather than due to a direct effect leading to blocking of Cdc2 phosphorylation of a specific substrate. One possibility would be inhibition of JNK activation in mitotic cells.

The co-transfection method described here is simple and possible to perform in larger series and promises to be useful for the characterization of apoptotic signaling pathways. It was particularly useful for paclitaxel-induced apoptosis, which is difficult to study. Taxol-induced increases in the caspase-mediated cleavage of the transfected marker (CK18) were inhibited by expression of Bcl-2. These results serve as a validation of the method and are consistent with previous findings on the role of Bcl-2 in taxol-induced apoptosis [23,24].

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